Estimating the Analytic Validity of Selected DNA Tests

Glenn E Palomaki, B.S.

Foundation for Blood Research

Scarborough, Maine

(207) 883-4131

palomaki@fbr.org

Analytic Validity of Selected DNA Tests

- General information about analytic validity
- Analysis of *CFTR* testing in prenatal screening
- Analysis of *HFE* testing for hereditary hemochromatosis
- Analysis of ‘sample mix-up’ rates in the ACMG/CAP proficiency testing program
- Status of analytic validity of DNA testing for breast/ovarian cancer and HNPCC

Analytic Validity

- Analytic sensitivity is the proportion of positive test results correctly reported by the laboratory among samples with a mutation(s) that the laboratory's test is designed to detect.
- Analytic specificity is the proportion of negative test results correctly reported among samples with no detectable mutation is present.
- Quality control assesses the procedures for ensuring that results fall within specified limits.
- Assay robustness is how resistant the assay is to changes in pre-analytic and analytic variables (e.g., sample degradation).

An ‘Optimal’ Dataset for Computing Analytic Sensitivity and Specificity

- An independent body establishes a sample set derived from the general population with selected ‘rare’ genotypes of interest according to disorder/setting criteria
- Samples also designed to test ‘robustness’
- This sample set is available for method validation by manufacturers via a consortium of laboratories
- Results are analyzed by the independent body and estimates provided

Available Sources of Data for Estimating Analytic Validity

- Method comparisons are of limited use
  - usually only two methods compared
  - pre-analytic errors may not be reported
  - small numbers of samples tested
  - ‘true’ genotype often not known
  - may not represent actual clinical practice
- External proficiency testing schemes are the only major reliable source currently available for computing analytic sensitivity and specificity

Data Source: ACMG/CAP MGL External Proficiency Testing Survey

- **Advantages**
  - Most clinical laboratories participate
  - Wide range of methodologies represented
  - Samples have confirmed genotypes
- **Disadvantages**
  - Over-representation of ‘difficult’ samples due to ‘educational’ nature of the program
  - Mixing of ‘screening’ and ‘diagnostic’ challenges
  - Limited number of DNA tests covered
  - Research laboratories, manufacturers, and laboratories outside the US participate
  - Artificial nature of sample preparation, shipping and handling

CFTR Analytic Validity Methodology: Analysis by Chromosome

Example 1:

Known genotype:	(delF508 / wild)
Laboratory result:	(wild / wild)
Interpretation:	false negative

Example 2:

Known genotype:	(delF508 / wild)
Laboratory result:	(G542X / wild)
Interpretation:	wrong mutation

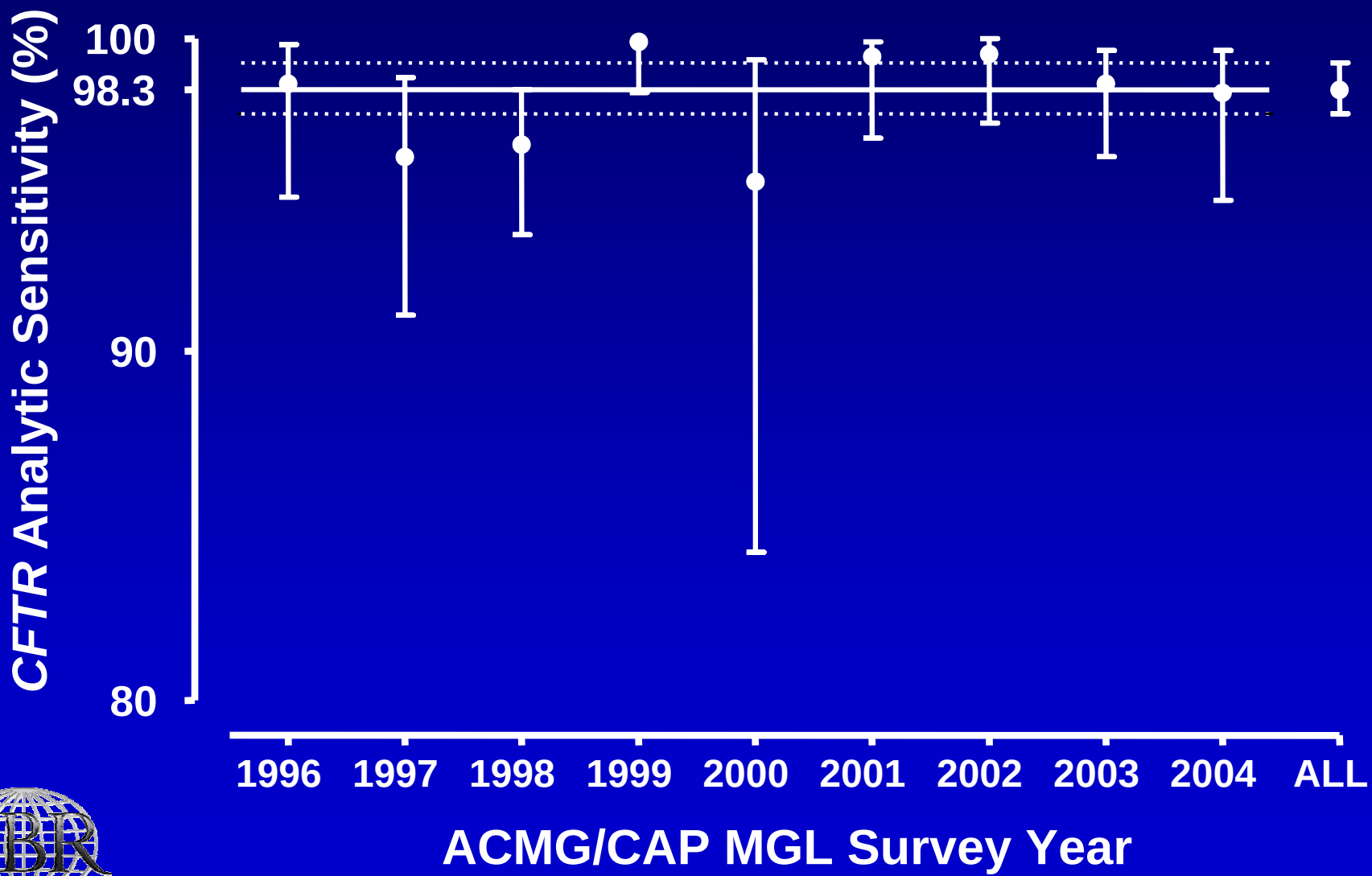
NEW DEFINITION: ‘Wrong mutation’ will be considered a ‘false positive’, since confirmatory testing might correct both types of errors.

Analytic Sensitivity: *CFTR* Mutations

Year	Chromosomes Challenged	True Positives	False Negatives	Analytic Sensitivity
1996	135	133	2	98.5
1997	128	123	5	96.1
1998	285	275	10	96.5
1999	212	212	0	100.0
2000	43	41	2	95.3
2001	168	167	1	99.4
2002	**196**	**195**	**1**	**99.5**
2003	**262**	**258**	**4**	**98.5**
2004	**163**	**160**	**3**	**98.2**
All	**1592**	**1564**	**28**	**98.3**

From ACMG/CAP MGL data - dell507 challenges removed

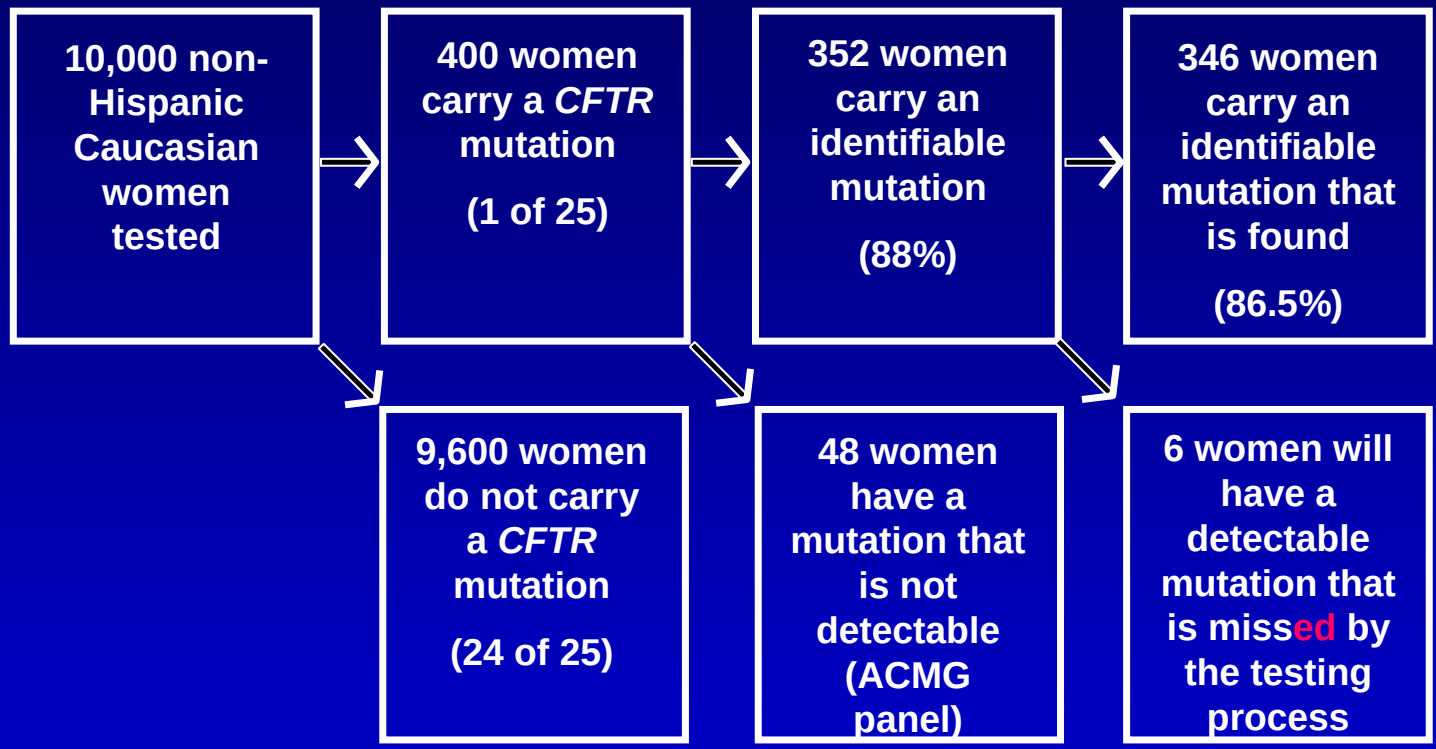
Analytic Sensitivity: *CFTR* Mutations



Analytic Sensitivity: *CFTR* Mutations

- **Analytic sensitivity is 98.3** (previously 97.9%)
  - based on up to 81 US laboratories (ACMG/CAP proficiency testing program)
  - estimate excludes three dell507 challenges
  - 95% confidence interval 97.5 to 99.2%
  - heterogeneous between 1996 and 2004
- **Gaps in knowledge**
  - method-specific analytic sensitivity
  - mutation-specific analytic sensitivity
  - 15 ‘ACMG’ mutations not included in external PT

Impact of Analytic Sensitivity on Prenatal Screening for Cystic Fibrosis



Analytic sensitivity of 98.3% reduces identification of *CFTR* mutation carriers from 88.0 to 86.5%, and detection of carrier couples from 77.4 to 74.8%.

Will an Affected Fetus be 'Missed' due to Analytic False Negatives?

- Most likely to be identified when a child whose parents had a negative prenatal screening test is diagnosed with cystic fibrosis and genotyped
- Estimated to occur about 1 per 154,000 couples tested
- One example has already been reported in the literature (Cunningham S *et al*., Arch Dis Child 1998;78:34508)
- Confirmatory testing is not helpful, as negative results are not subject to such efforts

Confidence in Analytic Sensitivity
Sample Size Estimates

- Target of 95% - rule out values below 80%
  - 190 of 200 mutations correct
- Target of 98% - rule out values below 90%
  - 196 of 200 mutations correct
- Target of 99% - rule out values below 95%
  - 198 Of 200 mutations correct
- Determining method- or mutation-specific analytic sensitivity might not be feasible for a single laboratory, but might be possible for a manufacturer via a consortium of laboratories

Analytic Specificity: *CFTR* Mutations

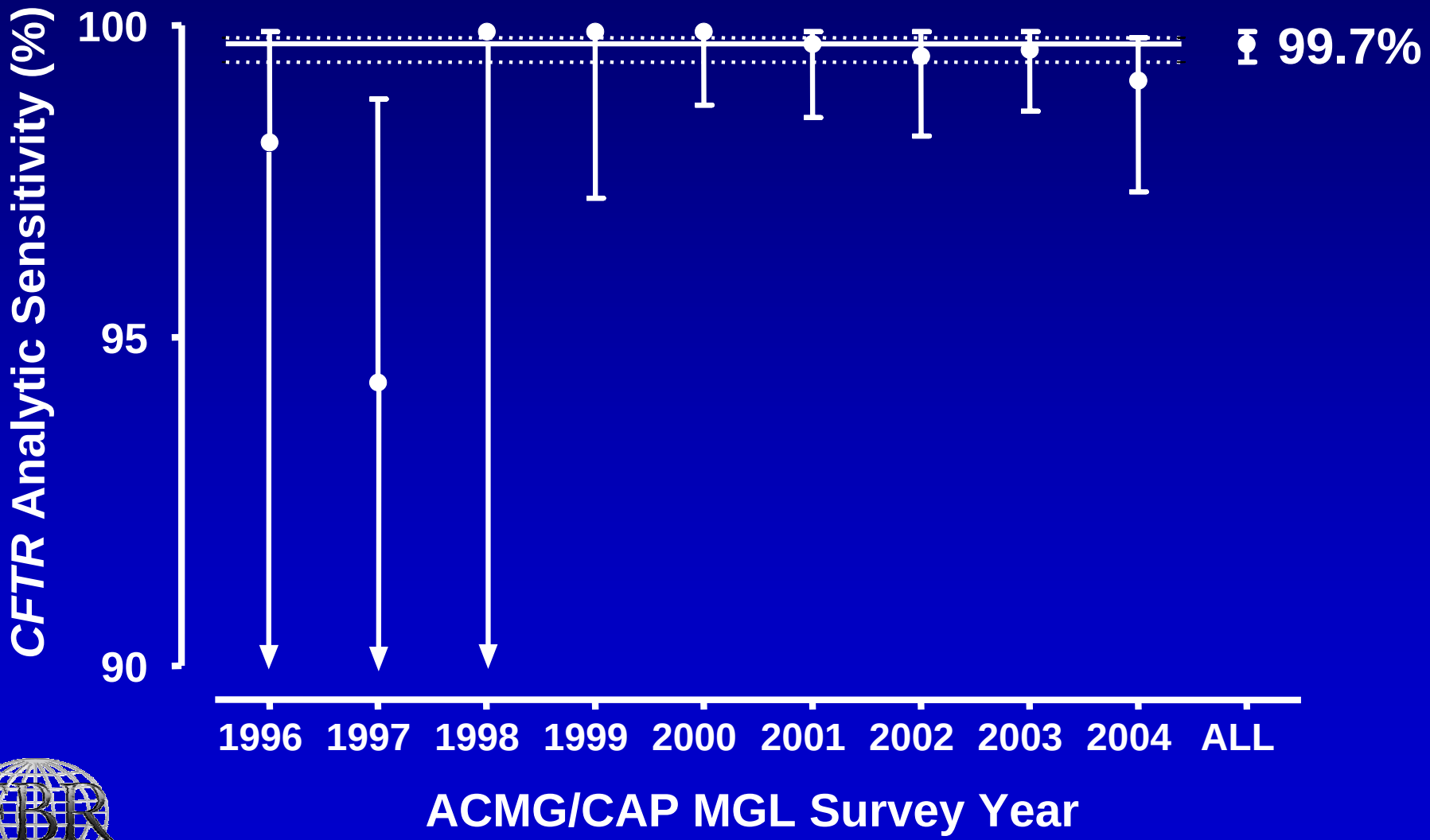
Year	Chromosomes Challenged	True Negatives	FP/ W Mut	Analytic Specificity
1996	53	52	1/0	98.1
1997	57	47	2/8	82.5
1998	21	21	0/0	100.0
1999	130	129	0/1	99.2
2000	273	273	0/0	100.0
2001	370	367	1/2	99.2
2002	**392**	**390**	**0/2**	**99.5**
2003	**526**	**524**	**2/0**	**99.6**
2004	**318**	**316**	**2/1**	**99.1**
All	**2141**	**2119**	**8/14**	**99.2**

ACMG/CAP MGL data, after removing 3 dell507 challenges

CFTR Analytic Specificity Needs Further Adjustment

- Too high a rate of ‘wrong mutation’ errors in the ACMG/CAP MGL survey because
  - to have a wrong mutation, a mutation must be present
  - a detectable mutation is uncommon in the population (1 in 60 chromosomes) but common in the survey (1 in 2 chromosomes)
- The rate of wrong mutations found in the survey should be ‘discounted’ by a factor of 30

Revised Analytic Specificity: *CFTR* Mutations



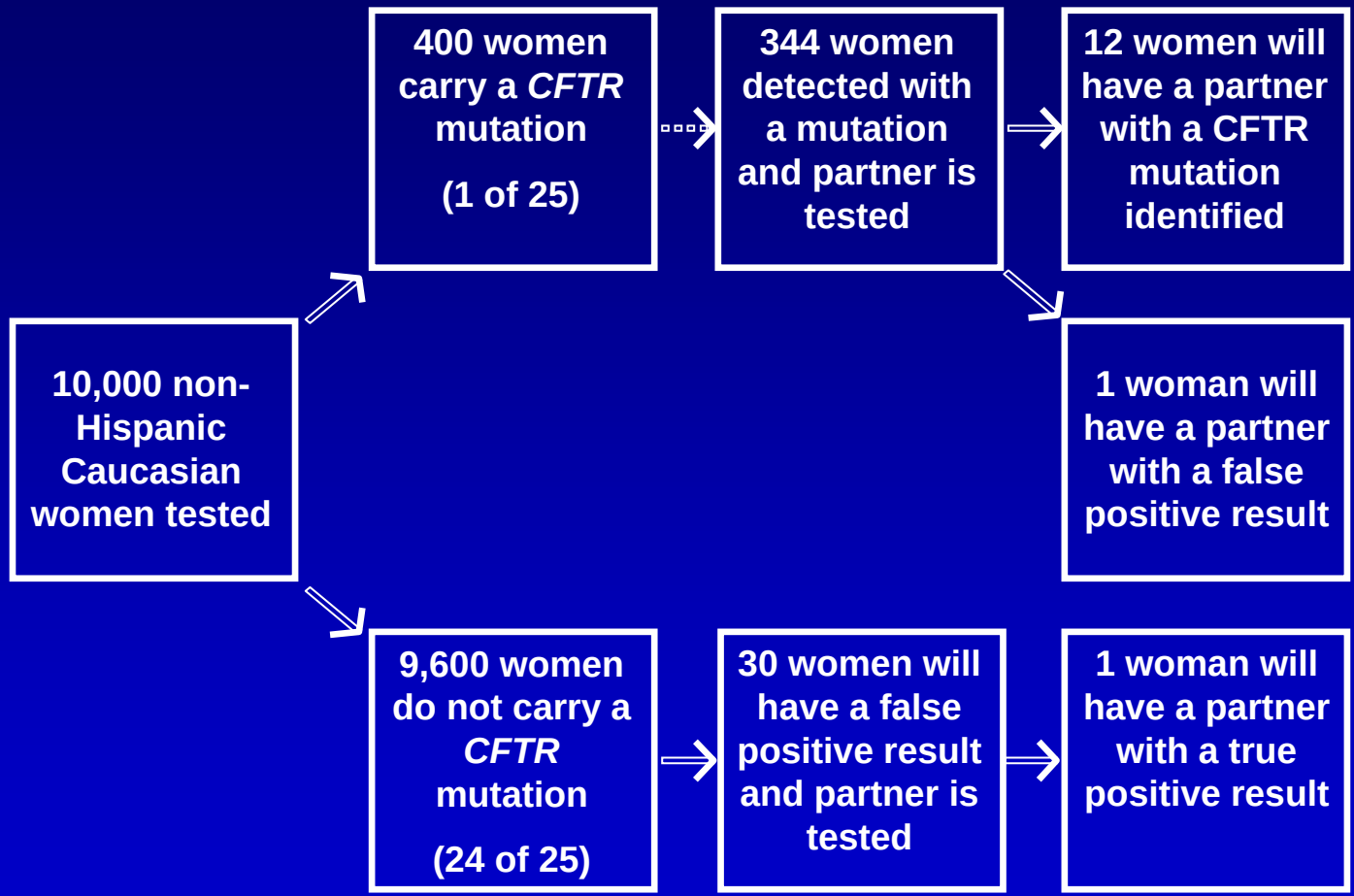
Analytic Specificity: *CFTR* Mutations

- **Analytic specificity is 99.7% (previously 99.4%)**
  - based on up to 81 laboratories (ACMG/CAP proficiency testing program)
  - estimate excludes dell507 challenges
  - the identification of a ‘wrong mutation’ (14) is more common than a ‘false positive’ (8), and this must be taken into account when estimating specificity
  - 95% confidence interval 99.4 to 99.9%
  - heterogeneous between 1996 and 2004
- **Gaps in knowledge**
  - method-specific analytic specificity
  - will a panel of more mutations have a different analytic specificity?

Confidence in Analytic Sensitivity
Sample Size Estimates

- Target of 98% - rule out values below 90%
  - 49 of 50 negative samples correct
- Target of 99.5% - rule out values below 98%
  - 398 of 400 negative samples correct
- Target of 99.9% - rule out values below 99.5%
  - 999 of 1000 negative samples correct
- Method-specific specificity is feasible only for a manufacturer via a consortium of laboratories

Impact of Analytic Specificity on Prenatal Screening for Cystic Fibrosis



An analytic specificity of 99.7% would result in 2 of 14 carrier couples being falsely identified.

How Often Will a Fetus be 'Missed' due to Analytic False Negatives?

- Most likely identified when a child whose parents had a negative prenatal screening test is diagnosed with CF and genotyped
- Estimated to occur about 1 per 154,000 couples tested
- One example has already been reported in the literature (Cunningham S *et al*., Arch Dis Child 198;78:34508)
- Confirmatory testing is not helpful, as negatives are not subject to such efforts

False Positive Carrier Couples?

- Are they as common as 2 of 14 (15%) of positive couples? (previously 4 of 16)
  - Routine confirmatory testing may identify some false positive couples before diagnostic testing is undertaken
  - A personal communication from a prenatal diagnostic laboratory confirms that false positive couples are undergoing amniocentesis (no firm estimate of prevalence)
  - Pilot trials found somewhat more than the expected 1 in 4 pregnancies affected (18 of 49)

Confirmatory Testing

Given that false positives/wrong mutations occur

- Confirmatory testing might be considered when any positive result is identified in:
  - an individual
  - a couple
  - a fetus
- Confirmatory testing could include:
  - repeating the test on the same sample
  - repeating the test on a different sample
  - performing a different assay on the same sample
  - performing a different assay on a different sample

Genetic Testing for Hereditary Hemochromatosis

- Mutations in the *HFE* gene are responsible for the majority (90%) of iron overload-related disease in Caucasians
- Homozygosity for the C282Y mutation is the most penetrant (5 to 10%) and account for 85 to 90% of clinically defined cases
- The H63D mutation is more common and far less penetrant
- Treatment (monitoring and phlebotomy) is likely to be effective if started early

Population Screening for C282Y Homozygosity

- Not currently recommended
- Aim of this analysis is to determine whether current analytic performance is sufficient
- Is confirmatory testing of homozygotes required?
- What is the possible impact of analytic errors on clinical validity?

ACMG/CAP Molecular Genetics Laboratory Survey

- Genotype results analyzed for data collected between 1998 and 2002
- Between 67 and 103 participating laboratories
- Both C282Y and H63D mutations challenged, but only C282Y analyzed
- Overall, 20 errors occurred in 2,043 laboratory genotyping challenges (1%)

HFE Analytic Validity Analyses are by Genotype not by Allele

Lab Result	Actual Genotype: 282/282	282/W	W/W
282/282	TP	FP	FP
282/W	FN	TN	TN
W/W	FN	TN	TN

282 = C282Y mutation, W = wildtype. H63D is ignored.

A Summary of ACMG/CAP Molecular Genetics Survey for *HFE* Testing

Lab Result	Actual Genotype 282/282	282/W	W/W
282/282	243	1	3
282/W	2	585	5
W/W	2	7	1,195

Analysis restricted to the C282Y mutation.

Estimating the Analytic Validity of Testing for C282Y Homozygosity

- Analytic Sensitivity
  - 243 of 247 true homozygote challenges correct
  - estimated sensitivity of 98.4%
  - 95 percent CI 95.9% to 99.4%
- Analytic Specificity
  - 1,792 of 1,795 true non-homozygote challenges correct
  - estimated specificity of 99.8%
  - 95 percent CI 99.4 to 99.9%

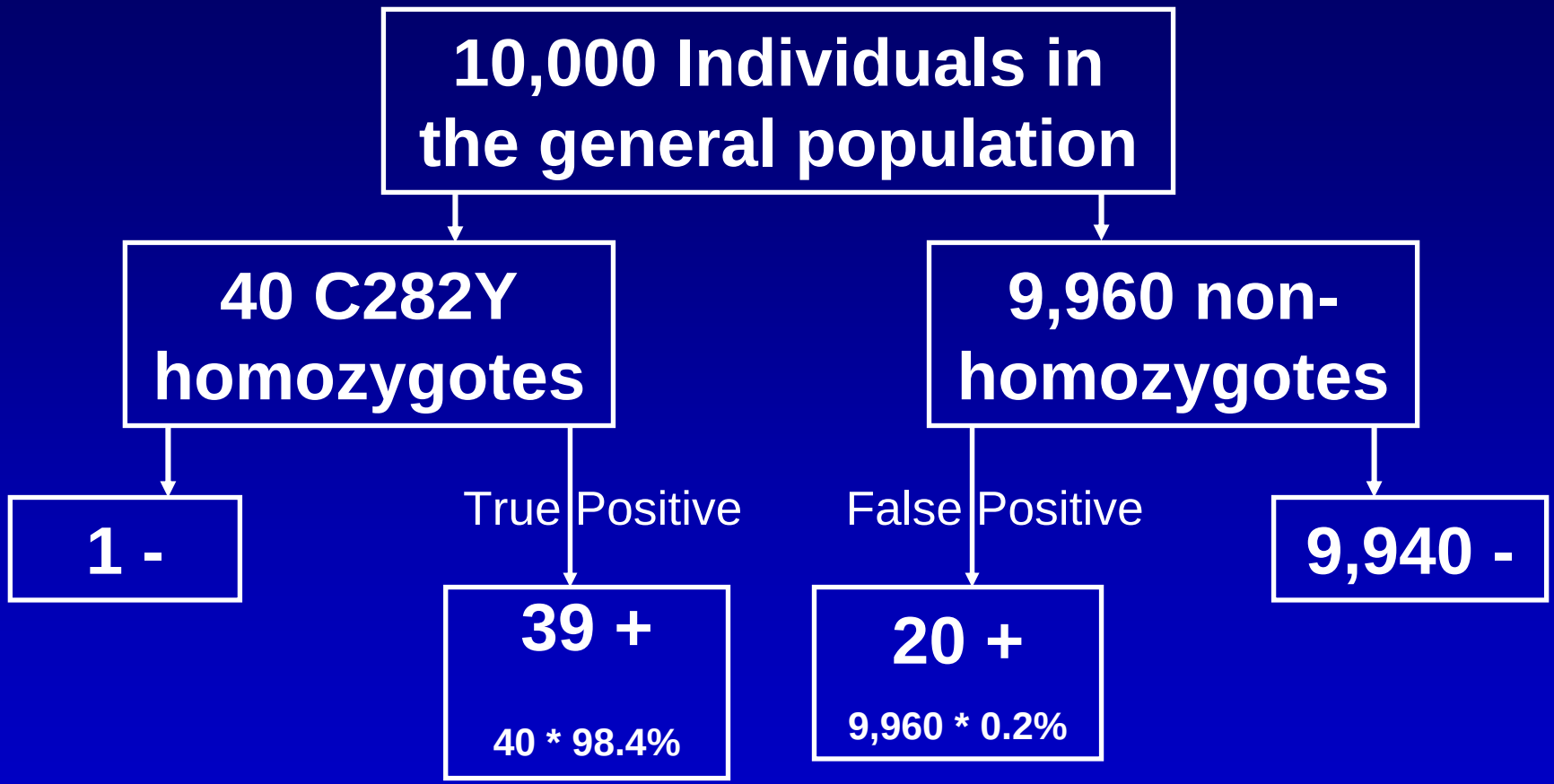
Too few challenges to determine whether these rates vary by year.

Analytic Positive Predictive Power

- Hypothetical population of 10,000 individuals (non-Hispanic Caucasians)
- Homozygous C282Y rate of 40/10,000
- Analytic sensitivity of 98.4%
- Analytic specificity of 99.8%

What proportion of those with a positive test result are true analytic positives?

Analytic Positive Predictive Power



Analytic PPV is 66% [39/ (39 + 20)]

Even with the high analytic performance for C282Y testing, one-third of those identified as homozygotes may be false positives. Confirmatory testing using a newly obtained sample may be warranted.

Additional Considerations

- Genotyping errors were made by labs that test only for C282Y as well as those testing for multiple mutations
- Errors occurred using several different methodologies
- None of the false positives were due to sample mix-up (a homozygous sample was not included)
- Errors were made by both clinical and non-clinical laboratories
- Errors were not due to a problem reported with a specific *HFE* primer
- A re-interpretation of previously reported screening results may be required
- Analytic positive predictive value lower in other racial/ethnic groups

Analysis of Sample Mix-up Rates in the ACMG/CAP MGL Surveys

- Sample mix-up rates are reported to be high in the factor V Leiden (FVL) / Prothrombin surveys
- Compare the rates for four surveys (*CFTR*, *HFE*, FVL and Pro) after accounting for
  - the number of participating laboratories
  - the proportion of identifiable sample mix-ups

Example of a Suspected Sample Mix-up

- Known *CFTR* genotypes distributed for testing
  - MGL-07 wild/wild
  - MGL-08 delF508/wild
  - MGL-09 G551D/wild
- Laboratory with suspected mix-up reports
  - MGL-07 delF508/wild
  - MGL-08 wild/wild
  - MGL-09 G551D/wild

Likely that this laboratory reversed the samples/results for MGL-07 and MGL-08

Observed Sample Mix-up Rates by Survey

Survey	Sample Challenges	Mix-ups	Rate (%)
FVL	4,038	9	0.22
Pro	3,555	7	0.20
HFE	2,461	4	0.15
CFTR	1,350	2	0.16
All	11,404	22	0.19

The Proportion of Detectable Sample Mix-ups Depends on the Challenges

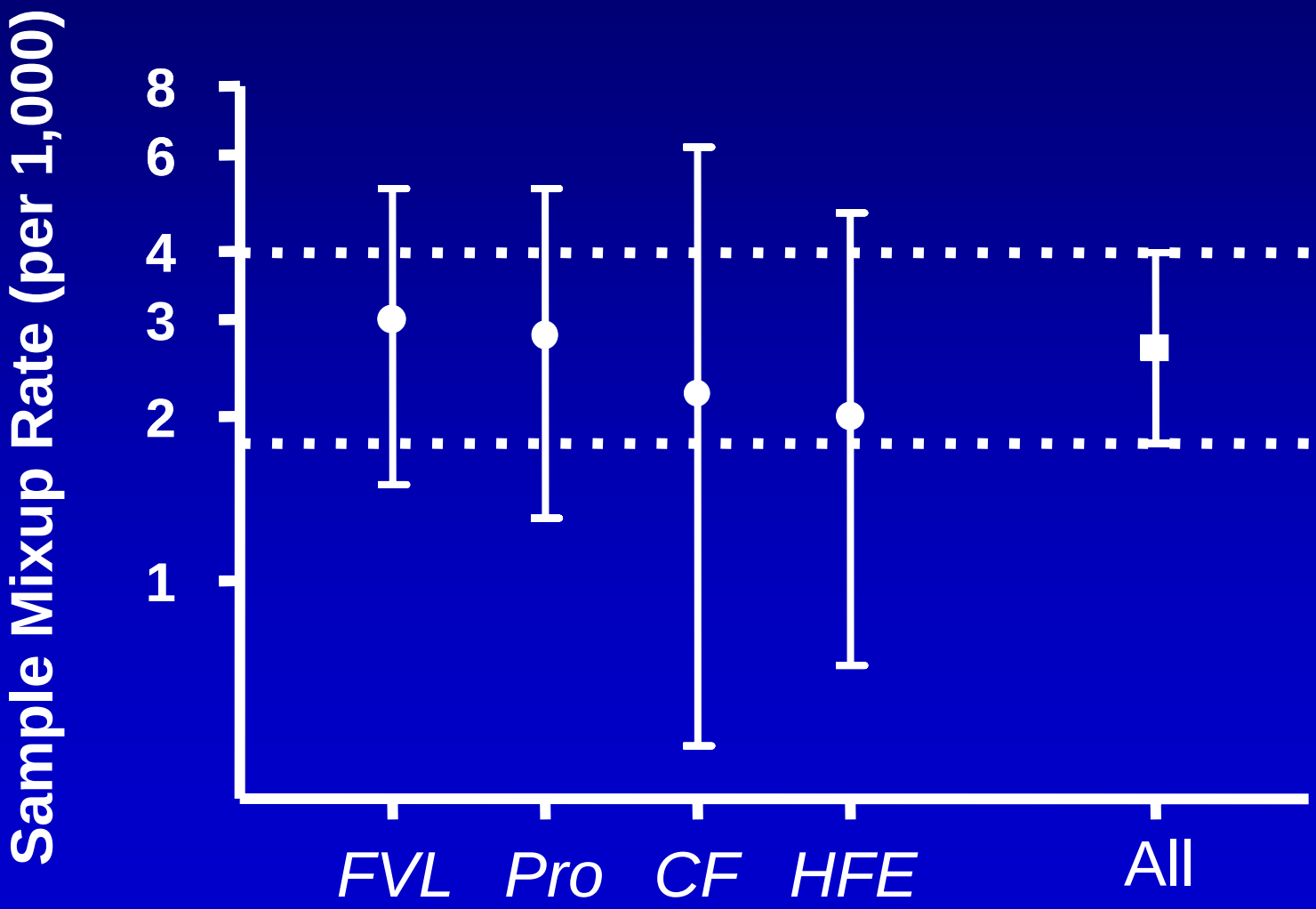
Example 1	Example 2	Example 3
R506Q / wild	R506Q / wild	wild / wild
R506Q / wild	wild / wild	R506Q / wild
R506Q / wild	wild/ wild	R506Q / R506Q
no mix-ups detected	two-thirds of mix-ups detected	all mix-ups detected

Sample Mix-up Rates by Survey

Survey	Challenges	Mix-ups	Rate (%)	
			Obs	Adj
FVL	4,038	9	0.22	0.30
Pro	3,555	7	0.20	0.28
HFE	2,461	4	0.16	0.18
CFTR	1,350	2	0.15	0.22
All	11,404	22	0.19	0.26

Adjusted Rate of Sample Mix-ups

ACMG/CAP MGL Surveys



Analytic Validity of *BRCA1/2* Mutation Testing for Hereditary Breast/Ovarian Cancer

- Reliable estimates are not possible due to
  - patent issues surrounding the *BRCA1* and *BRCA2* genes
    - only 1 U.S. laboratory can report clinical results
    - laboratories can license testing for three mutations
  - lack of appropriate proficiency testing for sequencing (only the three licensed mutations are currently challenged)

Analytic Validity of DNA Testing for Hereditary Non-Polyposis Colorectal Cancer (HNPCC)

- Involves sequencing of two or more genes (e.g., *MlH1*, *MSH2*)
- Several laboratories in the U.S. perform this testing, but no external proficiency testing is available
- Reliable estimates of analytic validity are not available

Acknowledgments

- Work was supported by a cooperative agreement with the CDC, Office of Genomics and Disease Prevention (CCU319352)
- The data sources for many of these analyses are the participant summary reports from the ACMG/CAP Biochemical and Molecular Genetics Resource Committee. We thank the committee members for their comments and hard work.